# SEMISYNTHETIC SYNTHESIS AND BIOLOGICAL ACTIVITY OF A CLOSTRIDIAL-TYPE FERREDOXIN FREE OF AROMATIC AMINO ACID RESIDUES

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SUMMARY: *Clostridium M-E* ferredoxin has been chemically modified by replacing the only aromatic amino acid residue it contains, $Tyr^2$, with leucine. The resulting *Clostridium M-E* [$Leu^2$]ferredoxin, which is devoid of aromatic amino acid residues, is as active as the native *Clostridium M-E* ferredoxin or as *Clostridium acidi-urici* ferredoxin as an electron carrier in the phosphoroclastic enzyme system.

Clostridial-type ferredoxins contain 54-56 amino acids and contain two $Fe_4S_4^*$ clusters. Tyrosine or other aromatic amino acids generally occur in two conserved positions, 2 and 30, in the peptide chain of clostridial-type ferredoxins (1), and have been implicated in the electron transfer function of these iron-sulfur proteins (2,3). We have recently described the preparation and properties of a derivative of *Clostridium acidi-urici* ferredoxin, [$Leu^2$]ferredoxin, in which a leucyl residue has been substituted for the tyrosyl residue in position 2 from the amino terminus (4). *C. acidi-urici* [$Leu^2$]ferredoxin is fully active as an electron carrier in two biological assays in which it was tested, and electron paramagnetic resonance experiments indicate that it accepts two electrons upon enzymatic reduction by pyruvate : ferredoxin oxidoreductase (4). Although these results indicate that the primary pathway of electron transfer in clostridial-type ferredoxins is not via Tyr or other aromatic amino acid residues, the fact that the modified ferredoxin still contained a Tyr residue in position 30 makes it difficult to exclude the possibility that this aromatic residue might function as the primary electron acceptor, and that intramolecular transfer between the two iron-sulfur clusters of clostridial-type ferredoxins could occur and explain the observed electron stoichiometry.

Abbreviations: BOC = *tert*-butyloxycarbonyl.

Although it has not previously been possible to replace the aromatic amino acid residue in position 30, modifications of $Tyr^{30}$ of *C. acidi-urici* ferredoxin were achieved by nitration of the aromatic residue of the apoprotein by conventional chemical procedures to yield [3-$NO_2$-$Tyr^{2,30}$]- and [$Leu^2$,3-$NO_2$-$Tyr^{30}$]apoferredoxins. However, after reduction of the nitro group to the amine function these derivatives could not be converted to ferredoxin derivatives of sufficient stability for testing in the enzymatic assays. [3-$NH_2$-$Tyr^{30}$]ferredoxin was sufficiently stable to permit testing, and was found to be fully active in the phosphoroclastic assay system.

The occurrence of a ferredoxin in *Clostridium M-E* with a single aromatic residue has recently been described (5). It contains its single aromatic residue, Tyr, in position 2 and arginine occurs in position 30 (5). It therefore appeared feasible to replace the aromatic residue in position 2 by an aliphatic amino acid residue through the use of chemical procedures similar to those previously described (4) and thereby obtain a clostridial-type ferredoxin entirely free of aromatic amino acid residues.

EXPERIMENTAL PROCEDURE:

The stock culture of *Clostridium M-E* was kindly provided by Dr. T.C. Stadtman. Bacterial cells were grown according to the procedure described previously (5). The ferredoxin was isolated from 73 g of cells by a modification of the procedure previously described (6), but it was found necessary to bring solutions of *Clostridium M-E* ferredoxin to about 90% saturation with ammonium sulfate in order to precipitate the protein.

*Clostridium M-E* ferredoxin was converted to the apoferredoxin (6), and the apoprotein was converted to the Boc-derivative by the following procedure: The apoprotein, 10 mg, in 2 ml of dimethylformamide (3 parts in 1 part water), adjusted to pH 9 to 10.5 by the addition of triethylamine (about 2 µl/ml), was treated with 10 µl of Boc-azide and was stirred at room temperature for 8 hours, and an additional 10 µl of the reagent was then added. After 20 hours, 3 µl of triethylamine and 5 µl of Boc-azide were

added. After a total of 24 hours, the solution was desalted, through the use of a column of Sephadex G-25, and lyophilized to yield 10 mg of *Clostridium M-E* Boc-apoferredoxin.

The Boc-apoferredoxin was dissolved in 1 ml of 1% ammonium bicarbonate and was treated with 25 µl of a solution of chymotrypsin (2 mg/ml) for 5.5 hours at 30 C, when another 25 µl of the chymotrypsin solution was added. After 12 hours, the solution was desalted by passage through a column of Sephadex G-25 and the water was removed by lyophilization. Amino acid analysis showed that the protein still contained about 0.3 moles of tyrosine per mole of apoferredoxin. The apoprotein was therefore redissolved in 1 ml of 1% ammonium bicarbonate to which was added 75 µl of the chymotrypsin solution and it was incubated at 37 C for 3 hours. The mixture was then desalted and lyophilized as described above, and 10 mg of protein was recovered.

The Boc-apoferredoxin, twice treated with chymotrypsin, was dissolved in 2 ml of dimethylformamide (3 parts in 1 part water) containing 2 µl/ml of triethylamine, and was coupled with Boc-Ala-Leu by means of its N-hydroxysuccinimide active ester as previously described (4). The *Clostridium M-E* [$Leu^2$]-apoferredoxin obtained was converted to the corresponding ferredoxin derivative by the addition of iron and sulfide under conditions for reconstitution (6) and was purified by chromatography on DEAE-cellulose (6). The *Clostridium M-E* [$Leu^2$]ferredoxin was precipitated with 90% ammonium sulfate.

Amino acid analyses and biological activities in the *Clostridium pasteurianum* phosphoroclastic system were determined as previously described (4).

RESULTS AND DISCUSSION:

The amino acid analysis of the *Clostridium M-E* ferredoxin isolated (Table I) agrees with that reported by Tanaka, *et al.* (5). Because of the presence of lysine residues in this ferredoxin, it was not feasible to remove the N-terminal amino acids by Edman degradation in order to subsequently replace the naturally occurring amino acid residues, as had been done previously with *C. acidi-urici* ferredoxin (4). Instead, the amino groups of the *Clostridium*

Table I Amino Acid Composition of Native *Clostridium M-E* Ferredoxin and Derivatives.

| | Protein | | |
|---|---|---|---|
| Residue | Native | Des-(Ala[1]-Tyr[2])-Apoferredoxin | [Leu[2]]-Ferredoxin |
| Aspartic acid | 7.82 (8) | 7.93 | 8.05 |
| Threonine | 1.90 (2) | 1.86 | 1.79 |
| Serine | 1.96 (2) | 2.04 | 1.92 |
| Glutamic acid | 5.11 (5) | 5.04 | 4.97 |
| Proline | 2.93 (3) | 3.37 | 3.00 |
| Glycine | 3.87 (4) | 3.97 | 3.88 |
| Alanine | 7.65 (8) | 6.95 | 8.57 |
| 1/2 Cystine* | 6.26 (8) | 6.02 | 5.55 |
| Valine | 4.46 (5) | 4.02 | 4.29 |
| Methionine | 0.0 (0) | 0.0 | 0.0 |
| Isoleucine | 4.61 (6) | 4.31 | 5.17 |
| Leucine | 0.0 (0) | 0.0 | 1.58 |
| Tyrosine | 1.09 (1) | 0.11 | 0.11 |
| Phenylalanine | 0.0 (0) | 0.0 | 0.0 |
| Lysine | 1.64 (2) | 1.60 | 1.65 |
| Histidine | 0.0 (0) | 0.0 | 0.0 |
| Arginine | 0.84 (1) | 0.82 | 0.88 |

Amino acid analysis was performed with a Beckman 117 automated analyzer using a single column procedure after hydrolysis of the proteins at 110 C for 22 hours in 6 N HCl. Iron and inorganic sulfide were removed from native and [Leu[2]]ferredoxin by precipitation of the proteins with trichloroacetic acid prior to analysis (6 ). The numbers of residues found in parentheses for native ferredoxin indicate the number of residues found in the amino acid sequence (5).

*Cysteine was determined in the normal hydrolysate. These values are variable and are approximately 75% of the values found for cysteic acid after performic acid oxidation of the sample.

*M-E* ferredoxin were protected by converting them to Boc-derivatives, and the two N-terminal amino acids were removed by treatment of the Boc-apoferredoxin with chymotrypsin, which is known to catalyze the hydrolysis of peptide bonds between the carboxy group of tyrosine, phenylalanine, or tryptophan and the amino group of the adjacent amino acid (7). Analysis of the product of the

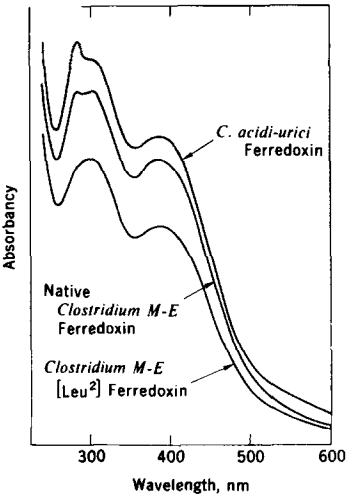


Fig. 1. Optical absorption spectra of native oxidized *Clostridium acidi-urici* ferredoxin, native oxidized *Clostridium M-E* ferredoxin, and oxidized *Clostridium M-E* [Leu$^2$]ferredoxin. Solutions approximately 20 μM with respect to ferredoxin were made in 0.01 M Tris·HCl buffer, pH 7.4.

chymotryptic digestion (Table I) demonstrates that an Ala and Tyr residue had been removed, although the apoprotein isolated still contained about 0.1 residue of Tyr. This finding confirmed the presence of the aromatic residue in position 2 from the N-terminal end as previously reported (5). The addition of the dipeptide Ala-Leu was accomplished by methods previously described and the amino acid analysis of the ferredoxin derivative isolated after reconstitution from the modified apoprotein (Table I) confirms its identification as *Clostridium M-E* [Leu$^2$]ferredoxin. The presence of somewhat more than 1 residue of leucine and 8 residues of alanine suggests that the amino groups in the native protein may not have been blocked completely as Boc-derivatives.

The absorption spectrum of *Clostridium M-E* [Leu$^2$]ferredoxin exhibits a single absorption maximum in the region of 280 nm and can be distinguished from the absorption spectrum of *Clostridium M-E* or *Clostridium acidi-urici* ferredoxins (Fig. 1). The spectrum of *Clostridium M-E* [Leu$^2$]ferredoxin is consistent with that expected for a clostridial-type ferredoxin free of aromatic amino acid residues. The absorption in the 280 nm region is a property of the iron-

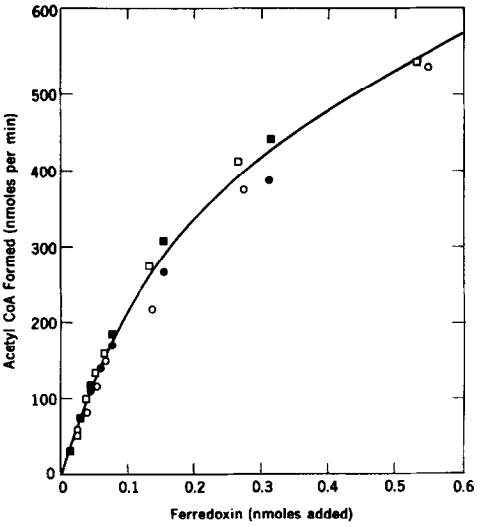


Fig. 2. Activity of native *C. acidi-urici* ferredoxin (●,▪), native *Clostridium M-E* ferredoxin (▫), and *Clostridium M-E* [Leu$^2$]ferredoxin (○) in the phosphoroclastic assay system. Circles and squares represent data obtained in the different experiments, and indicate the extent of variation observed with the control *C. acidi-urici* ferredoxin.

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sulfur chromophores. The presence of Tyr residues results in the appearance of additional absorption maximum at 280 nm as illustrated by the spectra of native *Clostridium M-E* and *C. acidi-urici* ferredoxins which contain one and two Tyr residues respectively (Fig. 1).

Solutions of native *Clostridium M-E* ferredoxin and native *C. acidi-urici* ferredoxins were about equally stable in air at neutral pH based on the stability of the absorption spectra. The *Clostridium M-E* [Leu$^2$]ferredoxin appears to be somewhat less stable than the native material under these conditions.

The activity of *Clostridium M-E* ferredoxin and *Clostridium M-E* [Leu$^2$]-ferredoxin are very similar and are indistinguishable from the activity of *C. acidi-urici* ferredoxin in the phosphoroclastic enzyme assay system (Fig. 2).

The observation that the activity of *Clostridium M-E* [Leu$^2$]ferredoxin cannot be distinguished from that of either native *Clostridium M-E* ferredoxin or of *Clostridium acidi-urici* ferredoxin in the phosphoroclastic enzyme assay

substantiates the previous conclusions that the aromatic residues commonly found in the clostridial-type ferredoxins do not have a primary role in electron transfer (4). Therefore, other routes of electron transfer, such as possibly via cysteinyl sulfur atoms, must be considered.

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